then changed to standard Iscove's growth medium or DMEM without thapsigargin, and cells were incubated for 2 or 4 hours at 37°C. Control cells underwent the same media changes but were not subjected to thapsigargin treatment.

Following the second incubation, the filter grown monolayers were washed once with phosphate buffered saline supplemented with calcium and magnesium (150 mM NaCl, 10 mM NaP_i, pH 7.4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$), after which they were fixed for 10 minutes in –20°C 100% methanol. Immunofluorescence labeling was performed using the well characterized 169 and 181 antibodies (gift of W. Guggino, Johns Hopkins University) directed against the R domain and the prenucleotide binding fold of the CFTR protein, respectively (Crawford *et al.*, Proc. Nat. Acad. Sci. 88:9262-9266 (1991)) and a monoclonal antibody directed against the α-subunit of the Na,K-ATPase (Gottardi and Caplan, J. Cell Biol. 121:283-293 (1993)).

Incubations with primary and rhodamine-conjugated secondary antibodies were performed as previously described (Gottardi and Caplan, Id.). Labeled cells were examined using a Zeiss LSM 410 laser scanning confocal microscope. All images are the product of 8-fold line averaging. Contrast and brightness settings were chosen so that all pixels were in the linear range. XZ cross sections were generated using a 0.2 μ motor step.

Results. To examine further the effects of thapsigargin on the subcellular distribution of the ΔF508 protein, we performed immunofluorescent localization of the CFTR protein in treated and untreated CFPAC cells. In untreated cells, CFTR staining is barely detectable in a diffuse cytoplasmic pattern surrounding the nucleus (Figure 4). This pattern is consistent with the localization of the ΔF508-CFTR protein to the ER in the untreated cells. In treated cells, viewed both *en face* and in XZ cross section, bright labeling of apical microvilli could be detected in most of the cells. Cells that were incubated for 2 hours following the thapsigargin treatment exhibited only apical staining. No intracellular ER labeling could be detected in these cells. Cells that were incubated for 4 hours following the thapsigargin treatment exhibiting CFTR staining both at the apical membrane and in the ER (data not shown). Thus, treatment with thapsigargin leads to redistribution of the mutant ΔF508-CFTR protein from the ER to the apical membrane.

As evidenced by the pattern observed in cells incubated for 4 hours after the removal of thapsigargin, ΔF508-CFTR protein synthesized following the removal of the drug is retained in the ER. These observations are consistent with the interpretation

that thapsigargin treatment permits mis-folded ΔF508-CFTR protein to be released from the ER and travel to its appropriate site of functional residence at the apical plasma membrane.

It is likely that the mechanism through which thapsigargin effects the redistribution of the ΔF508 CFTR protein from the ER to the cell surface is related to this compound's capacity to reduce the ER's intralumenal Ca^{++} concentration. It is also possible, however, that thapsigargin might interact directly with the ΔF508 CFTR protein to alter its tertiary structure. CFTR is related to the MDR family of ABC transport proteins. Members of the MDR family are capable of interacting with and transporting a wide variety of chemical compounds (Higgins, Ann. Rev. Cell Biol. 8:67-113 (1992)). It has been demonstrated that MDR proteins that carry mutations resulting in mis-folding and ER retention can be functionally rescued through exposure to compounds that are substrates for the particular MDR protein's transport activity (Loo and Clarke, J. Biol. Chem. 272:709-712 (1997); Loo and Clarke, J. Biol. Chem. 273:14671-14674 (1998)). Presumably, binding substrate compounds stabilizes the protein's conformation sufficiently to permit it to elude the ER's quality control machinery.

In light of the homology relating CFTR to the MDR proteins, it is possible that thapsigargin exerts its effect on ΔF508-CFTR through a similar mechanism. If CFTR manifests an MDR-like activity, thapsigargin could conceivably be a substrate analogue whose interaction with a binding site on CFTR could stabilize and modify this protein's structure. According to this model, thapsigargin's effect on calcium pumps and ER lumenal calcium concentrations would not be relevant to its mode of action in rescuing ΔF508-CFTR.

To test this possibility, we exposed ΣCFBE290⁻ cells to the calcium pump inhibitors DBHQ and cyclopiazonic acid, which are structurally unrelated to thapsigargin (Khan *et al.*, Biochem. 34:14385-14393 (1995); Whitcome *et al.*, Biochem. J. 310:859-868 (1995)). As assayed by immunofluorescence microscopy (data not shown), both compounds were able to recapitulate thapsigargin's capacity to induce ΔF508-CFTR surface delivery. Since DBHQ and cyclopiazonic acid are chemically quite distinct from thapsigargin and from each other, it is likely that their effects on ΔF508-CFTR arise from their shared capacity to release calcium from the ER lumen rather than from any direct interaction with the CFTR protein itself.

To ensure that thapsigargin-induced appearance of immunoreactive ΔF508-CFTR at the plasma membrane is due to the release of an ER retained cohort rather than to stimulation of new ΔF508-CFTR synthesis, protein synthesis was blocked during thapsigargin treatment and post-treatment chase periods through the addition of 10 mm cycloheximide. Inhibition of protein synthesis did not abrogate the thapsigargin effect (data not shown), demonstrating that thapsigargin releases a pre-synthesized pool of ΔF508-CFTR to the cell surface.

While not wishing to be bound by any theory, we speculate that thapsigargin exerts its effect by reducing the ER's intralumenal Ca^{2+} concentration, thus interfering with the functioning of calcium-dependent chaperone mechanisms. To establish whether the thapsigargin effect is indeed due to a reduction in intraorganellar Ca^{2+} rather than the consequent rise in cytosolic Ca^{2+}, we repeated the experiment in cells preloaded with BAPTA, which should chelate Ca^{2+} released into the cytosol by thapsigargin treatment (Tsien, R.Y., *Biochem.* 19, 2396 (1980)). The presence of BAPTA did not inhibit the thapsigargin-induced delivery of ΔF508-CFTR to the cell surface (data not shown), demonstrating that this effect is not due to increases in cytoplasmic Ca^{2+} concentration.

Experiment 4. Nebulized thapsigargin.

A nebulization chamber was constructed using an 8 quart plastic container with a lid that creates an air tight seal. A 'T piece nebulizer device' (Hudson RCI T-up Draft Nebumist Nebulizer) was inserted into the container via an opening located on the side of the chamber. The nebulization device was filled with 5 mls of 1 μM thapsigargin dissolved in physiologic saline solution. The gas source (high pressure air) was attached to the set up to create a flow rate of $\geq$12 liters per minute. Flow was adjusted to maintain a fine visible mist throughout the chamber. Numerous small ventilation holes were placed at the top of the chamber to ensure the escape of carbon dioxide. The nebulization chamber was kept in a fume hood during the experiments to allow for dispersion of any escaped mist.

Mice or cells were placed into the chamber prior to the onset of nebulization. Mice were observed continuously during the nebulization treatments and observations were documented every 15-30 minutes. Lungs were prepared for histologic analysis according to methods described previously (Courtois-Coutry *et al.*, Cell 90:501-510 (1997)).